Retinol esterification in cultured rat liver cells

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Retinol esterification was examined in cultured hepatocytes and stellate cells from the rat. Esterification of [^3H]retinol was linear for 2 h in both cell types. By increasing the concentration of retinol in the medium, there was a marked increase in retinol esterification in both cell types. The capacity for esterification of retinol was in the same order of magnitude in the two cell types at $3.5\text{ }\mu\text{M}$ -retinol in the medium. This represents a rate of retinol esterification which far exceeds that required to esterify the amount of retinol absorbed in the intestine. It was demonstrated in particulate homogenates from cultured hepatocytes that the esterification of retinol was dependent on acyl-CoA. Addition of 25-hydroxycholesterol or mevalonolactone promoted an increase in cholesterol esterification, whereas retinol esterification was unaffected, suggesting that cholesterol and retinol are esterified by two different enzymes. Some 80% of vitamin A in cultured hepatocytes is retinyl esters, mostly retinyl palmitate. By adding $87\text{ }\mu\text{M}$ -retinol in the medium the cells accumulated 100-fold free retinol and 2.5–3.0-fold retinyl esters within 1 h. When retinol-loaded cells were incubated without retinol, there was a marked decrease especially in free but also in esterified retinol. In the presence of 1 mM-oleic acid in the medium the amount of retinyl oleate was twice that in control cells.

Vitamin A (retinol) is absorbed in the small intestine and transported associated with chylomicrons (Goodman, 1980; Goodman *et al.*, 1965) via the intestinal lymph duct (Blomhoff *et al.*, 1984a). More than 95% of the absorbed retinol is esterified with long chain fatty acids in the intestinal mucosa and incorporated into the core of chylomicrons, where the esters stay until the chylomicron remnants are taken up by hepatocytes (Nilsson & Zilversmit, 1971; Lippiello *et al.*, 1981) via receptor-mediated endocytosis (Brown *et al.*, 1981). After internalization of the chylomicron remnants the retinyl esters are hydrolysed and re-esterified (Goodman, 1980). By experiments *in vitro* with homogenates from the intestine (Helgerud *et al.*, 1982, 1983), the mammary gland (Ross, 1982b) and the liver (Ross, 1982a) it is fairly well documented

that this esterification is catalyzed by acyl-CoA:retinol acyltransferase (ARAT), although it has been reported that cofactors were not required in experiments with liver (Futterman & Andrews, 1964) and retinal pigment epithelium (Berman *et al.*, 1980). It is not established whether retinol is esterified by the same enzyme that is responsible for cholesterol esterification (acyl-CoA:cholesterol acyltransferase, ACAT, EC 2.3.1.26), although recent data suggest that there exist two different enzymes for esterification of these two hydroxylipids (Ross *et al.*, 1984).

After uptake of chylomicron remnants into the hepatocytes there is a rapid intrahepatic transfer of retinol from the hepatocytes to stellate cells (Blomhoff *et al.*, 1982, 1984b). This transfer of retinol from hepatocytes to stellate cells takes place only if the vitamin A status is normal. Retinol is stored in the stellate cells (Blomhoff *et al.*, 1984b; Nakane, 1963; Wake, 1980). The biological mechanism for the transfer of retinol is unknown, but retinol esterification might be important in this process.

In the present paper we report data on esterifica-

Abbreviations used: ARAT, acyl-CoA:retinol acyltransferase; ACAT, acyl-CoA:cholesterol acyltransferase; DME medium, Dulbecco's modified Eagle's medium; DPPD, *NN'*-diphenyl-*p*-phenylenediamine.

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tion of retinol in hepatocytes and stellate cells. Furthermore, our data suggest that ARAT and ACAT are different enzymes.

Materials and methods

Chemicals

[9,10-³H]Oleic acid (sp. radioactivity 5.04 Ci/mmol) and [11,12-³H]retinol (sp. radioactivity 50 Ci/mmol) were obtained from Amersham International and [15-³H]retinol (sp. radioactivity 13.6 Ci/mmol) was purchased from New England Nuclear. DME medium supplemented with insulin (10 µg/ml), L-glutamine (0.1 mg/ml) and gentamicin (100 µg/ml) from Flow Laboratories was used as standard incubation medium for the cells.

Stellate cells were cultured in DME medium supplemented with 20% (v/v) fetal calf serum, penicillin/streptomycin (100 i.u./ml) and L-glutamine (0.6 mg/ml). Sterile plastic dishes from Costar, Cambridge, MA, U.S.A. were used to plate the hepatocytes and stellate cells (60 mm and 13 mm respectively). 25-Hydroxycholesterol was obtained from Steraloids, Wilton, NH, U.S.A. All other chemicals used were reagent grade and purchased from Sigma.

Isolation and culture of liver cells

Liver cells were prepared from male albino Wistar rats fed an ordinary low-fat diet and water *ad libitum* as outlined previously (Berry & Friend, 1969; Drevon *et al.*, 1977).

Hepatocytes were separated from non-parenchymal cells by differential centrifugation (50g for 30 s at 4°C) in a buffer containing 1% albumin. The hepatocytes were gently resuspended and washed by centrifugation and the viability was evaluated by the Trypan Blue (0.04%) exclusion test to be better than 95%. Contamination by nonparenchymal cells was less than 5%. By fluorescence microscopy (Blomhoff *et al.*, 1984b) we found that most of the nonparenchymal cells contaminating the hepatocytes contained large amounts of retinol and thus probably represented stellate cells.

The hepatocytes were finally plated in plastic dishes in DME medium containing gentamicin and 10% (v/v) calf serum (Drevon *et al.*, 1980a). After overnight incubation, at 37°C in an atmosphere of air/CO₂ (19:1) saturated with water, the medium was changed to a serum-free DME medium with 1% fatty acid-free albumin.

Stellate cells were isolated by a modification of the method described elsewhere (Knook *et al.*, 1982; De Leeuw *et al.*, 1984). After the combined Pronase/collagenase perfusion and incubation, 5 ml of the total non-parenchymal cell fraction was mixed with 7 ml of iso-osmotic Nycodenz solution (ρ 1.15 g/ml; Nyegaard, Oslo, Norway) and centri-

fuged at 1400g for 15 min. The floating layer, containing most of the stellate cells, was suspended in DME medium with 20% (v/v) fetal calf serum. About 250 000 stellate cells were seeded per well and the cells were identified by fluorescence microscopy. After 2 days in culture the cells were washed and the medium was changed every other day. When the cells were confluent after 2 weeks they were trypsin-treated, diluted 1:6 and replated. The cultures reached confluency about every week under these conditions.

Acylation pattern of the retinyl esters

Retinol dissolved in ethanol (87 µM final conc.) was added to hepatocytes in culture. After 1 h the medium was changed and the cells were washed six times in phosphate-buffered saline. Oleic acid (1 mM) bound to albumin (0.13 mM) was prepared (Nossen *et al.*, 1984) and added to the DME medium of some plates, whereas others were controls. After 2 h incubation at 37°C the cells were washed extensively and analysed for retinol and retinyl esters.

Ethanol (95% v/v) was added to the cells and vitamin A was extracted with hexane. Retinyl acetate was added as an internal standard. The hexane phase was dried by a stream of N₂ and the residue was dissolved in a small volume of chloroform/methanol (1:1, v/v) and analysed by h.p.l.c. (see below). External standards were used to identify the major peaks: retinol, retinyl acetate, retinyl palmitate, retinyl oleate and retinyl stearate.

Measurement of retinol esterification

Hepatocytes were incubated in DME medium for various periods in the presence of [11,12-³H]retinol (usual sp. radioactivity 286 d.p.m./pmol, 3.5 µM final concn.) dissolved in ethanol or dimethyl sulphoxide (1% final concn.). The medium and cells were harvested as described in detail elsewhere (Nossen *et al.*, 1984).

After centrifugation of the medium to remove cells, 1 ml of the medium was extracted by 20 vol. of chloroform/methanol [2:1 v/v (Folch *et al.*, 1957)] and analysed by using aluminium oxide columns and h.p.l.c. (described below) to identify different metabolites of retinol. The cells were centrifuged in ice-cold phosphate-buffered saline, resuspended in water and frozen at -20°C. Samples (15 µl) were removed in duplicate after vortex-mixing for protein determination (Lowry *et al.*, 1951) using bovine albumin as standard.

Homogenates of cultured hepatocytes (Dounce homogenizer) were freed of soluble fractions by centrifugation at 160 000g for 30 min. The pellet was resuspended in potassium phosphate buffer

(0.2M, pH 7.4) and incubated with 80 μM -[15- ^3H]retinol, 20 μM -palmitoyl-CoA, 15.4 μM -bovine serum albumin, 5mM-dithiothreitol and 12.3 μM -DPPD (Rasmussen *et al.*, 1984). Retinol and DPPD were added dissolved in dimethyl sulphoxide. The incubation was started by adding the particulate fraction from the cells (100–140 μg) after the other components had been preincubated in a shaking water bath at 37°C for 5 min. The final incubation volume was 250 μl . After 3 min the reaction was stopped with ethanol. Some samples were incubated without activated palmitic acid to measure retinol esterification independent of ARAT. Samples to which ethanol was added at time zero were used as blanks and all samples were extracted into 5 ml of hexane. After phase separation the organic phase was evaporated under N_2 and labelled retinol was separated from retinyl esters by h.p.l.c. (see below) by injecting the lipid residue dissolved in chloroform/methanol (1:1, v/v). Based on radioactivity in the two fractions, retinol esterified (%) was calculated and retinol esterification was expressed as nmol of ester formed/min per mg of protein.

Separation and quantification of retinol derivatives

After extraction the retinol derivatives were separated either on alumina columns or by h.p.l.c. Alumina columns were used to separate retinyl esters from retinol by eluting with hexane/diethyl ether, 49:1 (v/v) and 1:1 (v/v) respectively (Helgerud *et al.*, 1982). Most of the alumina column separations of retinol derivatives were verified by h.p.l.c. identification.

The h.p.l.c. detectors were a model 440 monitoring at 313nm and a model 481 Lambda Max monitoring at 328nm, both from Waters Associates.

For analysis of the acylation pattern in the retinyl esters a Supelco-sil LC-8 column (25cm $\times$ 4.6mm i.d.) from Supelco, Bellefonte, PA, U.S.A. was eluted with water/methanol (3:37, v/v) at a flow rate of 1.5ml/min. Integration was performed by a SP 4270 Integrator (Spectra Physics, San Jose, CA, U.S.A.).

For separation of radiolabelled retinol and the total retinyl ester fraction a Lichrosorb RP-8 standard column (Merck, Darmstadt, Germany) was eluted with water/methanol (9:191, v/v) at a flow rate of 1ml/min. Fractions (1ml) were collected and retinol was eluted in fraction 4–6, whereas the esters were in fractions 13–14.

Radioactivity was quantified by liquid scintillation using Instagel II (Packard) as scintillator.

Measurement of cholesterol esterification

This was performed in cultured hepatocytes as described previously (Drevon *et al.*, 1980a,b).

[^3H]Oleic acid was bound to fatty acid-free bovine serum albumin in a molar ratio of 1:1 (Nossen *et al.*, 1984). The cholesteryl esters were isolated by t.l.c. and quantified by liquid scintillation (Drevon *et al.*, 1980a).

All experiments were performed with at least triplicate dishes and the experiments were performed at least three times with similar results. The data are presented as means $\pm$ s.d. for triplicate samples.

Results

Retinol esterification in cultured hepatocytes

In the presence of 3.5–35 μM of retinol in the medium there was a linear esterification of retinol for 60–120 min (Fig. 1). The accumulation of free retinol was more rapid and reached a plateau after 15–30 min. Approx. 20% of the cell-associated radioactivity was metabolized to water-soluble derivatives (Fig. 1). Retinol dissolved in ethanol or dimethylsulphoxide was taken up and esterified to the same extent by the hepatocytes (results not shown).

With increasing concentration of retinol in the medium there was an increase in the cellular accumulation of esterified retinol up to about 90 μM of free retinol in the medium (Fig. 2). Although we are aware that our experimental system is rather unphysiological, we used 3.5 μM (= 1 $\mu\text{g}/\text{ml}$) of free retinol in the medium in most of the following studies since the concentration of retinol in blood plasma is of this order of magnitude (Peterson *et al.*, 1973).

Fig. 1. Time course of uptake and esterification of radio-labelled retinol in cultured hepatocytes

At 1 day after plating the cells were given DME medium containing [^3H]retinol (sp. radioactivity 286 d.p.m./pmol, 3.5 μM final concn.). The cells were incubated for different periods of time, washed six times, harvested and total radioactivity (Δ), free retinol ($\circ$) and retinyl esters ($\bullet$) were measured in the cells. The derivatives were separated by h.p.l.c.

Fig. 2. Dose-response of esterification of retinol in cultured hepatocytes

The cells were exposed to increasing amounts of [^3H]retinol (sp. radioactivity 2.9–286 d.p.m./pmol) for 20 min under the same incubation conditions as described in the legend to Fig. 1. Retinyl esters were quantified after alumina column chromatography.

The time of culture for the hepatocytes from isolation to 28 h after plating did not significantly affect retinol esterification (results not shown).

Retinol esterification in homogenates of hepatocytes

Retinol esterification in homogenates of hepatocytes was 0.24 ± 0.05 nmol/min per mg of cell protein ($n=5$) after 1 day in culture. Similar activities were also found in freshly isolated hepatocytes. There was very low or no retinol esterification in the homogenates without addition of acyl-CoA derivative of the fatty acid. The value obtained in the absence of palmitoyl-CoA was 0.02 ± 0.02 nmol/min per mg of protein.

Retinol esterification in cultured stellate cells

At $3.5 \mu\text{M}$ free retinol in the medium there was an increasing accumulation of total retinol (free and ester radioactivity) in the cells with increasing time (Fig. 3a). The retinol esterification was linear up to at least 2 h (Fig. 3b). With increasing concentration of retinol in the medium there was a marked and almost linear increase in retinol esterification in stellate cells comparable with what was found in hepatocytes (Fig. 4). The retinol esterification per mg of cell protein was in the same order of magnitude as for the cultured hepatocytes at low concentrations (0.35 – $18 \mu\text{M}$) of retinol in the medium, whereas there was a somewhat higher esterification in stellate cells at $175 \mu\text{M}$. Since the contaminating 2–5% of stellate cells in our cultured hepatocytes represent less than 0.5% of the cell protein these data indicate that both hepatocytes and stellate cells have the capacity to esterify

Fig. 3. Time course of uptake and esterification of retinol in cultured stellate cells

The cells were cultured overnight and incubated in DME medium with [^3H]retinol, (sp. radioactivity 286 d.p.m./pmol, $3.5 \mu\text{M}$ final concn.). After different periods of time the cells were washed six times and harvested; total retinol radioactivity (free and ester) and retinyl esters were separated on alumina columns.

retinol. The ability to esterify retinol in stellate cells was similar after 1 day and 5 weeks in culture (results not shown).

Comparison of ARAT and ACAT in hepatocytes

Cholesterol esterification, as evaluated by incorporation of [^3H]oleic acid into cholesteryl esters, increased 8-fold during 60 min incubation of the cells with 25-hydroxycholesterol ($25 \mu\text{M}$), whereas retinol esterification was unaffected (Figs 5a and 5b). Mevalonolactone (1 mM) promoted a rapid 4-fold increase in cellular cholesterol esterification and there was no increase in retinol esterification after 60 min incubation as compared with the control (results not shown).

Acylation pattern of retinyl esters

About 80% of the total amount of lipid-soluble vitamin A derivatives are retinyl esters in primary cultures of hepatocytes (Table 1). Palmitic acid

contributes about 75% of the fatty acids in the total retinyl ester fraction, whereas oleate and stearate make up about 4 and 10% of the retinyl esters, respectively (line *a*). After exposure of the cells to a large dose of retinol for 60 min the amount of free retinol increased 100-fold, whereas the amount of retinyl esters increased 2.5–3.0-fold (Table 1, line *b*). Retinyl palmitate accounted for the largest increase in the amount of esters, but retinyl oleate represented the largest fractional increase (6-fold).

After loading the cells with retinol the cells were washed and incubated in the absence and presence of 1 mM-oleic acid for 2 h (Table 1, lines *c* and *d*, respectively). In the absence of retinol in the

medium there was a 50% decrease in the amount of esterified retinol. The amount of retinyl palmitate and retinyl stearate was decreased to the same extent whether oleate was present or not. However,

Fig. 4. Dose-response of esterification of retinol by cultured stellate cells

Stellate cells were cultured for 3 weeks and the esterification of retinol was measured in confluent cells. The cells were incubated in DME medium containing [³H]retinol (sp. radioactivity 6.2–3086 d.p.m./pmol, final concn. 0.35–175 μM) for 60 min and the radioactivity incorporated into the retinyl esters was measured.

Fig. 5. Retinol and cholesterol esterification in cultured hepatocytes

At 1 day after plating the medium was replaced with DME medium containing [³H]retinol (sp. radioactivity 286 d.p.m./pmol, 3.5 μM final concn.) (*a*) or [³H]oleic acid (11.3 d.p.m./pmol, 50 μM final concn.) (*b*). In some of the media 25-hydroxycholesterol (25 μM, ○) was added, whereas other dishes were controls (●).

Table 1. Amount of free retinol and retinyl esters in cultured hepatocytes

(*a*) The cells were analysed 20 h after plating in DME medium containing 10% (v/v) calf serum. (*b*) After 20 h in culture the medium was changed to DME containing 87 μM-retinol and incubated for 60 min more. Some plates were then exposed to a new medium without (*c*) and with (*d*) 1 mM-oleic acid for another 120 min. Three of the retinyl esters were identified and the rest of them made up for the difference to the total retinyl esters. The cultures contained 2% of stellate cells and the analyses were performed by h.p.l.c. as described in the Materials and methods section.

Conditions	Amount (nmol/mg of cell protein)				
	Free retinol	Total retinyl ester	Retinyl esters		
			Palmitate	Oleate	Stearate
(a) Standard medium	0.16 ± 0.08	0.61 ± 0.21	0.52 ± 0.06	0.03 ± 0.01	0.07 ± 0.02
(b) 87 μM-retinol for 1 h	18.6 ± 5.2	2.0 ± 0.3	1.09 ± 0.06	0.18 ± 0.02	0.20 ± 0.01
(c) DME for 2 h	3.39 ± 0.33	1.11 ± 0.16	0.72 ± 0.01	0.07 ± 0.01	0.13 ± 0.02
(d) 1 mM-oleic acid for 2 h	1.66 ± 0.08	1.15 ± 0.25	0.72 ± 0.02	0.15 ± 0.01	0.14 ± 0.01

in the presence of oleate the mass of retinyl oleate in the cells (Table 1, line *d*) was similar to what was found in retinol-loaded cells (Table 1, line *b*). It should also be noted that the amount of free retinol in cells incubated in the absence of oleate was higher than in cells incubated with oleate.

Discussion

Our data clearly demonstrate that hepatocytes as well as stellate cells are able to esterify retinol. The daily requirement of vitamin A in a 250g rat is about 9 µg (30 nmol) (Mallia *et al.*, 1975). Extrapolating from our cell culture experiments we find that the hepatocytes and the stellate cells are capable of esterifying 1000-fold and 100-fold, respectively, more retinol than the daily requirement for vitamin A. Although there are many uncertainties in calculations based on experiments *in vitro*, it seems safe to conclude that both cell types of major importance for retinol metabolism in the liver have the ability to esterify retinol in physiological concentrations.

It is possible to keep stellate cells in culture for several weeks by applying normal cell culture techniques. In spite of the fact that several different ways to purify hepatocytes after collagenase perfusion were applied, we never completely eliminated stellate cells from our hepatocyte preparations. This could be due to a close anatomical relationship *in vivo*.

The question whether retinol and cholesterol are esterified by the same enzyme has been raised by several authors (Helgerud *et al.*, 1982; Ross, 1982a; Ross *et al.*, 1984; Norum *et al.*, 1983). By feeding experiments in rats it was demonstrated that ACAT activity was altered without parallel effects on retinol esterification (Norum *et al.*, 1983). Ross *et al.* (1984) showed that an acylamide (58-035) selectively inhibited cholesterol esterification both in cultured hepatoma cells and microsomal fractions, whereas retinol esterification was unaffected. We now demonstrate that retinol esterification is unaltered during stimulation of cholesterol esterification by 25-hydroxycholesterol or mevalonolactone (Drevon *et al.*, 1980a,b). All these data indicate that the liver has two separate enzymes for esterification of retinol and cholesterol.

By increasing the concentration of retinol in the media we demonstrated increased retinol esterification as evaluated by isotope studies (Figs. 2 and 4) as well as by mass measurements (Table 1, lines *a* and *b*). Retinol dissolved in ethanol gave a very marked increase in the amount of free retinol in the cells, whereas a 2.5–3.0-fold increase in retinyl esters was seen. This suggests that ARAT in

cultured hepatocytes normally does not work at $V_{\max}$.

When the cells were exposed to a retinol-free medium after loading there was significant decrease of the amount of total retinyl esters from 2.0 to 1.11 nmol/mg of cell protein (Table 1, lines *b* and *c*). This finding is consistent with the view that stored retinyl esters have to be hydrolysed when retinol is removed from the medium, although the possibility of loss of retinyl ester due to other phenomena such as oxidation has not yet been ruled out.

The release of vitamin A from the liver probably depends on retinyl ester hydrolase (Smith *et al.*, 1978). Since there are different hepatic enzymes for both production and hydrolysis of retinyl esters, a continuous esterification and hydrolysis may take place in steady state, as shown for cholesterol and cholesteryl esters in cultured macrophages (Brown *et al.*, 1980). On the other hand, the situation could be quite different for vitamin A because the stellate cells are important for storage of retinyl esters in the liver. Thus, it may be that the retinyl esters stored in stellate cells are turning over at a different rate than retinyl esters in the hepatocytes. In fact a recent kinetic study suggests that there are two pools of vitamin A, one small pool involved in a rapid turnover, and one larger pool involved in more long term storage (Lewis *et al.*, 1981).

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